Published in final edited form as:
Neurosci Lett. 2013 October 25; 555: . doi:10.1016/j.neulet.2013.07.053.

Independent of 5-HT1$_A$ receptors, neurons in the paraventricular hypothalamus mediate ACTH responses from MDMA

Dmitry V. Zaretsky[a], **Maria V. Zaretskaia**[a], **Joseph A. DiMicco**[b], **Pamela J. Durant**[a], **Christian T. Ross**[a], and **Daniel E. Rusyniak**[a,b]

[a]Department of Emergency Medicine, Indiana University School of Medicine, Indianapolis, IN, 46202, United States

[b]Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, IN, 46202, United States

Abstract

Acute and chronic complications from the substituted amphetamine 3,4-methylenedioxymethamphetamine (MDMA) are linked to activation of the hypothalamic-pituitary-adrenal (HPA) axis. How MDMA activates the HPA axis is not known. HPA responses to stress are known to be mediated through the paraventricular (PVH) hypothalamus and to involve serotonin-1a (5-HT1$_A$) receptors. We sought to determine if the PVH and 5-HT1$_A$ receptors were also involved in mediating HPA responses to MDMA. Rats were pretreated with either saline or a 5-HT1$_A$ antagonist, WAY-100635 (WAY), followed by a systemic dose of MDMA (7.5 mg/kg i.v.). Animals pretreated with WAY had significantly lower plasma ACTH concentrations after MDMA. To determine if neurons in the PVH were involved, and if their involvement was mediated by 5-HT1$_A$ receptors, rats implanted with guide cannulas targeting the PVH were microinjected with the GABA$_A$ receptor agonist muscimol, aCSF, or WAY followed by MDMA. Compared to aCSF microinjections of muscimol significantly attenuated the MDMA-induced rise in plasma ACTH (126 vs. 588 pg/ml, P=<0.01). WAY had no effect. Our data demonstrates that neurons in the PVH, independent of 5-HT1$_A$ receptors, mediate ACTH responses to MDMA.

Introduction

In humans and laboratory animals, MDMA activates the hypothalamic-pituitary-adrenal (HPA) axis thus increasing circulating concentrations of adrenocorticotropic hormone (ACTH) and corticosteroids [11, 29]. This activation has been linked to both acute and chronic complications. Acutely, corticosteroids facilitate the development of hyperthermia from MDMA. Rats which have had their adrenal or pituitary removed fail to develop, or have attenuated, hyperthermia [7, 38]. Alterations in the function of the HPA axis are also associated with chronic complications from MDMA. Compared to drug-naive subjects, habitual MDMA users have higher basal plasma ACTH concentrations and blunted stress responses [8]. Similar effects have been reported in persons with clinical depression and anxiety [19] linking the HPA axis to psychiatric problems in heavy MDMA users [26].

Corresponding Author: Daniel E. Rusyniak, MD, Indiana University School of Medicine, Department of Emergency Medicine, 1050 Wishard Blvd, Rm 2200, Indianapolis, IN, USA 46202, Business: 317-630-7276, drusynia@iupui.edu.

Despite a connection between the HPA axis and its complications, how MDMA activates the HPA axis is not known. Experimental stress activates the HPA axis similar to that seen with MDMA [5]. These responses are suppressed, or prevented, by inhibiting neurons in the region the paraventricular hypothalamus (PVH)[5]. 5-HT1$_A$ receptors are also linked to stress responses: blocking 5-HT1$_A$ receptors, through systemic or intracerebral administration of 5-HT1$_A$ antagonists, reduces increase in ACTH from restraint stress [21, 22].

The PVH and 5-HT1$_A$ receptors may also play a role in MDMA's ACTH responses. MDMA is a potent serotonin releaser and activates neurons in the PVH [12, 14]. Furthermore, microinjecting, or perfusing, 5-HT1$_A$ agonists into the PVH increases plasma ACTH concentrations [2, 31, 32] and conversely, microinjections of the 5-HT1$_A$ antagonist WAY-100635 (WAY) into the PVH inhibit plasma ACTH increases evoked by the systemic administration of 8-OH-DPAT [31]. Based on this, we hypothesized that 5-HT1$_A$ receptors in the PVH were involved in ACTH responses to MDMA.

Materials and Methods

Animals

Male Sprague-Dawley rats (weight 300 ± 20 g; Harlan, Indianapolis, IN) were singly housed, maintained in a 12 h light/dark cycle beginning at 7 a.m, and fed ad libitum. Experiments were conducted in isolated rooms between 10:00 a.m. and 2:00 p.m. Animals were acclimated to the experimental rooms for a minimum of 2 h before experiments. Care and use of rats was approved by the Indiana University Animal Care and Use Committee.

Chemicals

MDMA, generously provided by the NIH, was dissolved the day of the experiment in normal saline; injection volumes were 1 ml/kg body weight. Muscimol (Sigma-Aldrich, St. Louis, MO) was dissolved in artificial CSF (aCSF) and stored at −20°C until the time of the experiment. WAY (Sigma-Aldrich) was dissolved in sterile saline and used fresh or stored until use at −20°C.

Surgical preparation

Animals were anaesthetized with a ketamine/xylazine cocktail (80 mg/kg ketamine; 11.5 mg/kg xylazine, i.p.), with supplemental doses provided as needed, femoral artery (for blood withdrawal) and venous (for drug administration) catheters were inserted as previously described [1, 36]. For experiments involving microinjections, animals were placed in a stereotaxic apparatus and bilateral microinjection guide cannulas (26 gauge, Plastics One, Roanoke, VA) were positioned into the region of the PVH using the following coordinates: incisor bar 5 mm above the interaural line, 10 degree angle from the sagittal plane, AP 0.0 mm; LR ±2.1 mm; HD −7.3 mm. Once inserted, cannulas were secured with stainless steel screws, Vetbond glue (3M, St. Paul, MN) and cranioplastic cement. Dummy-wire cannulas were inserted in the guides, and the rats returned to their home cages for a minimum of seven days.

ACTH Measurement

Blood (0.35 ml) was withdrawn from arterial catheters and replaced with an equal volume of sterile saline. The syringes used for blood draws contained 60 μl of a solution containing 10 mg/ml EDTA and 50% aprotinin. Samples were immediately transferred to 0.5 ml Eppendorf tubes, and centrifuged at 12,100 *g* for 26 sec in a MiniSpin centrifuge (Eppendorf, Hamburg, Germany) to separate the plasma. Samples were stored at −80°C until analyzed.

Plasma ACTH concentrations were determined by RIA using a modification of a previously reported method [43]. This double antibody assay uses a rabbit antiserum (IgG Corp, Nashville, TN) recognizing the $ACTH_{5\text{-}18}$, and goat anti-rabbit globulin (EMD Millipore, Billerica, MA) to precipitate the antigen antibody complex. ACTH for RIA standards was from Bachem (Torrance, CA), and ^{125}I-ACTH from Diasorin Inc (Stillwater, MN). Counts were recorded on a gamma counter (Cobra II, Perkin Elmer, Watham, MA) and duplicate standard curves were used.

Experimental Protocols

Time Course of ACTH after MDMA

We determined peak plasma concentrations of ACTH by taking blood samples from rats (n=4) 5 min before and 15, 30, 60, 120, and 180 min after an i.v. dose of MDMA (7.5 mg/kg). This MDMA dose causes temperature and cardiovascular responses mimicking those seen in human intoxication, results in plasma concentrations similar to humans taking recreational doses [10], and, microinjections of muscimol into the region of the DMH (a brain region associated with sympathetic responses to MDMA) attenuated temperature, cardiovascular, and behavioral responses produced by this dose [36]. Since intraperitoneal injections cause stress responses—including increases in plasma ACTH concentrations [27]—drugs were injected intravenously.

The effect of a systemic dose of WAY on MDMA-induced increases in plasma ACTH

To determine WAYs effects on plasma ACTH after MDMA, rats (n=4) were pretreated (i.v.) with equal volumes of vehicle (0.9% NaCl) or WAY (0.5 mg/kg) followed 5 min later by MDMA (7.5 mg/kg). After a 2-day washout animals were crossed-over to receive the other pretreatment followed by MDMA; the order of administration was random. Blood was withdrawn 5 min before WAY or saline and 15 min after MDMA. The WAY dose was the same we previously showed decreases MDMA locomotion [35] and prevents increases in plasma ACTH produced by the 5-$HT1_A$ agonist 8-OH-DPAT [41]. Although 8-OH-DPAT is both a 5-$HT1_A$ and 5-HT7 agonist, only its 1A effects are linked to ACTH secretion[6]. Like many drugs WAY is selective but not specific for certain receptors. At high doses WAY antagonizes dopamine-4 (D4) receptors [25], however the concentration we used how is below that shown to block D4 receptors *in vivo* [25]

Effect of muscimol or WAY microinjections on increases in plasma ACTH produced by MDMA

To investigate whether the PVH, and more specifically 5-$HT1_A$ receptors in the PVH, play a role in endocrine responses to MDMA, we conducted the following experiment. Rats were acclimated to their cage (Raturn®, Basi, Layfayette IN) overnight. Then, microinjectors (33 gauge, Plastics One) were connected to a 10 μl Hamilton syringe with Teflon tubing (ID 0.12 mm; OD 0.65 mm; BASi). The syringe was mounted on an infusion pump (Model 200, KD Scientific, Holliston, MA) to microinject either 100 nL of aCSF, muscimol (80 pmol), or WAY (0.5 nmol). Muscimol, a $GABA_A$ agonist, was used to suppress neuronal activity in the PVH where it has been shown to hyperpolarize neurons [16]. The dose used has been shown to decrease ACTH concentrations caused by stress [40]. The dose of WAY was the same we have previously shown prevents hypothermia, when microinjected into the medullary raphe pallidus, from a systemic dose of 8-OH-DPAT [35]. Solutions contained 0.25% fluorescent microspheres (0.04 μm in diameter; Invitrogen, Carlsbad, CA) to mark the injection site. Since placing microinjectors evokes a mild stress response, animals were left undisturbed for at least 1 h after the injectors were placed [44]. Blood was withdrawn to obtain baseline ACTH concentrations, then 5 min later animals were microinjected, over 30

seconds, with muscimol, WAY, or aCSF and five min later MDMA (7.5 mg i.v.) was injected.

Verification of microinjections

At the conclusion of each experiment rats were injected with pentobarbital (100 mg/kg, i.v.) and transcardially perfused with 30 ml of cold saline (4°C) followed by 30 ml of 4% paraformaldehyde. Brains were removed and placed overnight (at 4°C) in 4% solution of paraformaldehyde and afterwards stored in a 30% sucrose solution until processed.

Using a cryostat (Leica Microsystems, Buffalo Grove, IL) 40 μm coronal brain sections were cut and microsphere location was determined using a fluorescent microscope according to the atlas of Paxinos and Watson (47) by an observer blinded to group allocation.

Data Analysis and Statistical Procedures

Statistics and graphs were generated using Prism (Graphpad Software, San Diego, CA) software. ACTH was reported as change from baseline. In the time course experiment we ran a repeat measures ANOVA test with a Dunnett's post-hoc test comparing each value to baseline. Experiments with systemic WAY were analyzed using a paired Student t-test and microinjection experiments analyzed by ANOVA with a Tukey's HSD post-hoc analysis. In all of these experiments significance was defined as a P-value <0.05.

Results

ACTH Time course andthe effect of systemic WAY

An i.v. dose of MDMA caused a rapid increase in plasma ACTH concentrations (Fig. 1A). Peak concentrations occurred at 15 min and were >1000% above baseline. Mean baseline and peak ACTH concentrations (± 1 SEM) were 72 (±11) and 893 (±167) pg/ml respectively.

WAY (0.5 mg/kg, i.v.) significantly decreased plasma ACTH concentrations evoked by MDMA (Fig 1B). Mean increases in ACTH (± 1SEM) in the WAY and Saline groups were 198 (±52) and 642 (±66) pg/ml respectively. Mean baseline concentrations (± 1SEM) for the WAY and saline groups were 55 (±6) and 66 (±15) pg/ml.

Microinjection sites

Microinjection sites were confirmed by fluorescent microspheres as demonstrated in the representative brain section (Fig. 2A). The approximate centers of each microinjection were elucidated from the corresponding H&E stained section (Fig. 2B) and plotted on schematic coronal sections in which the PVH was demarcated using drawings adapted from the atlas of Paxinos and Watson [33]. The total area encompassing all the injections for the PVH is shown in gray (2C). Injection sites extended in this region so that correct locations were attributed to injections between −1.8 to −2.12 mm (from bregma) in the region of PVH.

Effect of muscimol or WAY microinjections on increases in plasma ACTH produced by MDMA

Compared to aCSF, microinjections of muscimol into the PVH, but not WAY, significantly reduced increases in plasma ACTH evoked by MDMA (Fig. 3). There were no differences in mean baseline concentrations between aCSF (65±17 pg/ml), muscimol (57±17 pg/ml), or WAY (74±21 pg/ml) respectively. We have previously shown that microinjections of muscimol or aCSF do not by themselves significantly increase ACTH [1]. It is not known however whether microinjections of WAY into the region of the PVH affect ACTH

concentrations, therefore, we conducted an additional experiment microinjecting either aCSF or WAY into the PVH followed by a systemic dose of saline (n=4/group). There were no differences in mean change in plasma ACTH (48±8 pg/ml v 43±8 pg/ml, p=0.4) or in baseline ACTH concentrations (50±7 pg/ml v 32±13 pg/ml).

Discussion

Our results show that 5-HT1_A receptors and neurons in the region of the PVH are involved in HPA activation produced by MDMA, but that the location of the involved 5-HT1_A receptors is outside of the PVH.

Serotonin receptors are known to regulate plasma ACTH concentrations [23], and drugs that activate either 5-HT1_A [18, 21, 23] or 5-HT2_A [18, 34] receptors increase circulating concentrations of ACTH. A potent releaser of serotonin, MDMA has effects on body temperature that are thought to involve 5-HT1_A and 5-HT2_A receptors [15, 30]. Both receptors could mediate the neuroendocrine effects of MDMA. 5-HT1_A agonists (i.e., 8-OH-DPAT) cause increase in ACTH concentrations that closely parallel those seen with MDMA (Fig. 1A) [18]. While we confirmed a role for 5-HT1_A receptors in ACTH responses to MDMA, our findings do not eliminate the additional involvement of 5-HT2_A receptors. In support of this, systemic injections of WAY reduce but do not abolish MDMA-induced increases in plasma ACTH (Fig. 1B). Data from Nash et al. supports a role for 5-HT2_A receptors as they showed that ketanserin and mianserin, at relatively high doses, decreased corticosterone concentrations after MDMA [29]. While both drugs antagonize 5-HT2_A receptors they also antagonize other monoaminergic receptors including alpha-1 receptors, which are linked to hypothalamic control of ACTH secretion [20]. Nash's study also seems to conflict with our WAY experiments. They reported that pindolol, a 5-HT1_A antagonist, did not prevent MDMA-induced increases in corticosterone [29]. Pindolol, however, has been shown to be a mixed 5-HT1_A agonist-antagonist [17], exhibiting both agonist [4, 39] and antagonist [13, 42] properties depending on dose and local serotonin concentrations. Nash used a dose of pindolol (0.3 mg/kg) below that shown to have 5-HT1_A antagonist properties [13, 42].

The finding that the PVH was involved in MDMA-mediated ACTH responses suggests that MDMA activates neurons in the PVH—the primary site of corticotropin-releasing hormone synthesis[24]. Previous work has shown that 5-HT1_A receptors in the PVH mediate ACTH responses to both systemically and centrally administered 5-HT1_A agonists [2, 31, 32]. When viewed in this light, we were surprised to find that 5-HT1_A receptors in the PVH did not mediate ACTH responses from MDMA. One possible explanation for this is that we microinjected insufficient concentration of WAY effectively block 5-HT1_A receptors. This however is unlikely as the dose of WAY was the same as that which when microinjected into the medullary raphe pallidus prevents hypothermia induced by a systemic dose of 8-OH-DPAT [35]. Rather, we suggest that the location(s) of 5-HT1_A receptors involved in MDMA-mediated effects on plasma ACTH are upstream of the PVH. There are numerous potential sites that send efferents to the PVH and which contain, or are known to be influenced by 5-HT1_A receptors: e.g., dorsal *raphe* nucleus, the bed nucleus of the stria terminalis, the preoptic area of the hypothalamus, and the amygdala [9, 22, 37]. Many of these sites are also known to send GABAergic projections to the PVH [28], such that through the release of 5-HT, MDMA could disinhibit CRH neurons in the PVH. Any of these, or numerous other, sites could be involved. Another possibility is that WAY acted on 5-HT1_A receptors at the level of the pituitary [3]. Since our data showed nearly complete suppression (78%) when the PVH was inhibited we think this possibility is unlikely. Further work is needed to determine the site and mechanism of WAY's action on increases in plasma ACTH seen after administration of MDMA.

In conclusion, we have shown that neurons in the PVH are involved in to the activation of the HPA axis mediated by the substituted amphetamine MDMA. Furthermore we have shown that 5-$HT1_A$ receptors outside of the PVH are involved. Understanding how the HPA axis is activated by MDMA and other amphetamines may shed light on the mechanisms behind their acute and chronic complications.

Acknowledgments

Research reported in this publication was supported by the National Institute on Drug Abuse of the NIH under award number R01DA026867. Furthermore, this work was conducted in a facility constructed with support from the National Center for Research Resources, of the NIH under award number C06 RR015481-010. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Abbreviations

MDMA	3,4-methylenedioxymethamphetamine
HPA	hypothalamic-pituitary-adrenal axis
ACTH	adrenocorticotropin releasing hormone
5-HT1A	serotonin-1a receptors, PVH, paraventricular hypothalamus
PVH	paraventricular hypothalamus
WAY	WAY-100635
aCSF	artificial cerebrospinal fluid

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Highlights

- MDMA increases plasma ACTH levels
- Systemic WAY-100635 prevents these ACTH increases
- Inhibiting of neurons in the PVH also prevents these ACTH increases
- Microinjections of WAY-100635 into the PVH do not prevent ACTH increases
- Activation of HPA axis by MDMA separately involves 5-HT1a receptors and PVH neurons

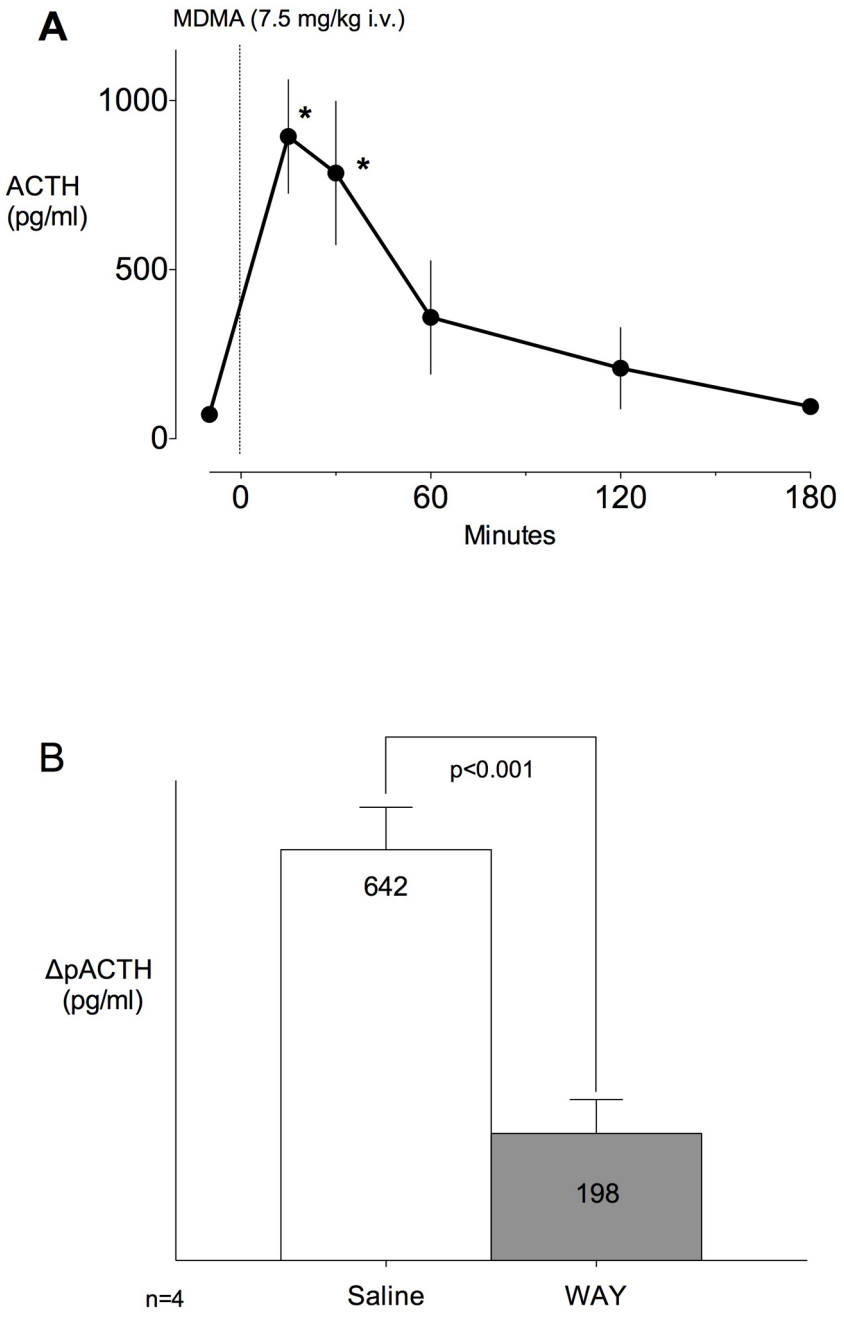

Fig 1.

Fig. 1A. Intravenous injection of MDMA causes a rapid and significant rise in ACTH. Circles represent mean ACTH concentrations and correspond to the time points when blood was withdrawn for measurement (−10, 15, 30, 60, 120, and 180 min). Error bars represent 1 SEM. *Represent significant difference compared to baseline ACTH concentrations as determined by repeat measures ANOVA with a Dunnett's post hoc analysis. Fig. 1B. A systemic dose of WAY attenuates increases in ACTH produced by MDMA. Vertical rectangular bars, and their corresponding numbers, represent the mean change in plasma ACTH after a dose of MDMA (7.5 mg/kg) for animals previously injected with WAY (0.5

mg/kg, i.v.) or saline. Error bars represent 1 SEM. The *P*-value for comparison between the WAY and Saline groups is represented (paired t-test).

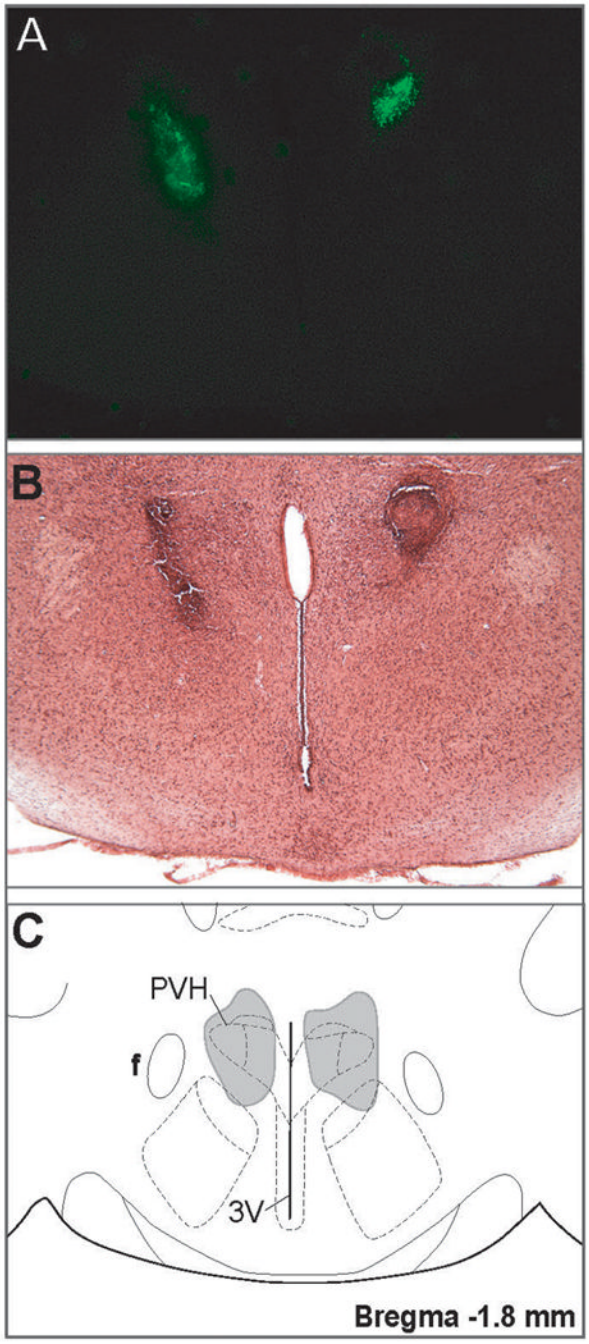

Fig. 2.

Injection sites. Figure A and B show the injection site of a single, representative, animal. The distribution of green fluorescent microspheres (A) marks the sites of a muscimol injection into the region of PVH. The neutral red counterstained section (B) shows the anatomic landmarks used for marking the injection sites. Figure 3C is a schematic, adapted from the atlas of Paxions and Watson [33], summarizing the microinjection sites for studies employing muscimol, WAY, and aCSF. The gray schematic in each figure represents the cumulative spread of fluorescent microspheres co-microinjected with drug or aCSF. Each region shown is a representative; injections may have extended anteriorly or posteriorly by

no more than 0.5 mm. Abbreviations: PVH=paraventricular hypothalamus, f=fornix, 3V=3rd ventricle.

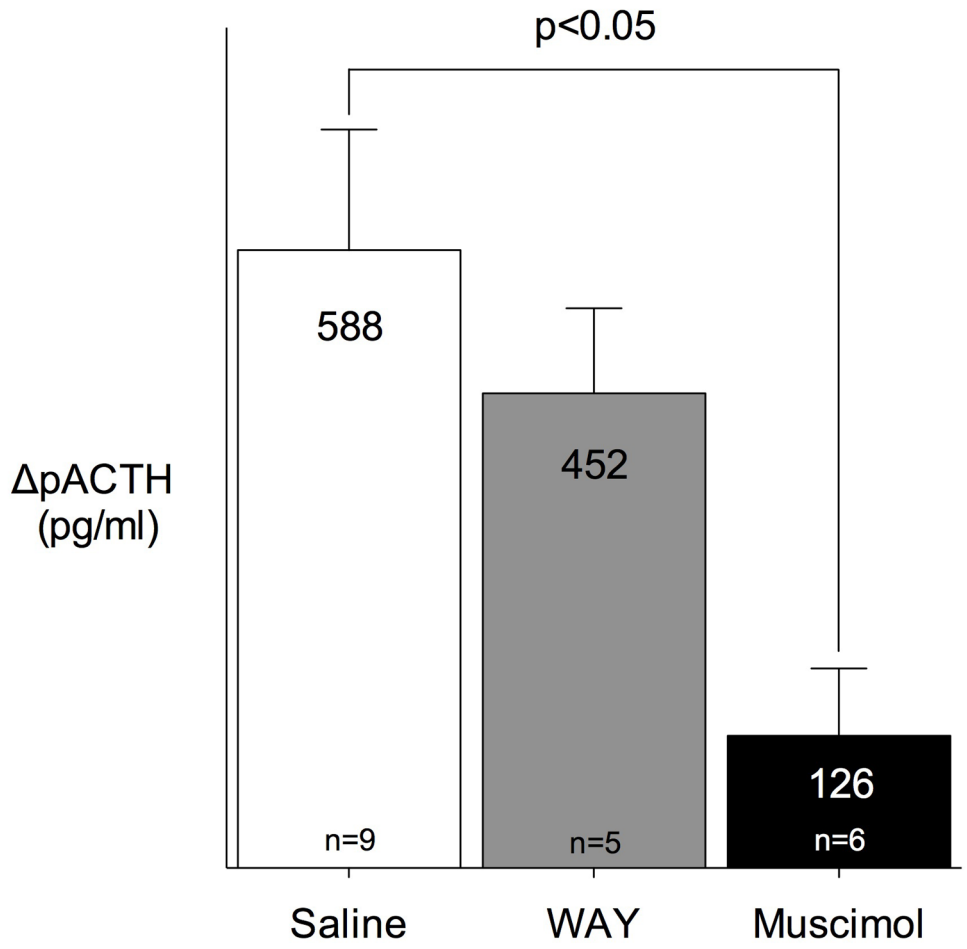

Fig. 3.

The effect of microinjections of muscimol, WAY, or aCSF into the PVH on MDMA-mediated increases in ACTH: Vertical rectangular bars and their corresponding numbers represent the mean change in plasma ACTH after a dose of MDMA (7.5 mg/kg) in animals previously microinjected into the PVH, with either aCSF, muscimol (80 pmol/100nl) or WAY (0.5 nmol/100nl). Error bars represent 1 SEM. The number of animals in each group is noted by the number at the base of each vertical rectangular bar. *P*-values were calculated using ANOVA with a Tukey's HSD post hoc analysis.